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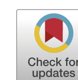
REVIEW

Recommendations for the use of the acetaminophen hepatotoxicity model for mechanistic studies and how to avoid common pitfalls



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KEY WORDS

Acetaminophen hepatotoxicity;
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Abstract Acetaminophen (APAP) is a widely used analgesic and antipyretic drug, which is safe at therapeutic doses but can cause severe liver injury and even liver failure after overdoses. The mouse model of APAP hepatotoxicity recapitulates closely the human pathophysiology. As a result, this clinically relevant model is frequently used to study mechanisms of drug-induced liver injury and even more so to test potential therapeutic interventions. However, the complexity of the model requires a thorough understanding of the pathophysiology to obtain valid results and mechanistic information that is translatable to the clinic. However, many studies using this model are flawed, which jeopardizes the scientific and clinical

Abbreviations: AIF, apoptosis-inducing factor; AMPK, AMP-activated protein kinase; APAP, acetaminophen; ARE, antioxidant response element; ATG, autophagy-related genes; BSO, buthionine sulfoximine; CAD, caspase-activated DNase; CYP, cytochrome P450 enzymes; DAMPs, damage-associated molecular patterns; DMSO, dimethylsulfoxide; EndoG, endonuclease G; FSP1, ferroptosis suppressing protein 1; *Gclc*, glutamate−cysteine ligase catalytic subunit; *Gclm*, glutamate−cysteine ligase modifier subunit; GPX4, glutathione peroxidase 4; GSH, glutathione; GSSG, glutathione disulfide; HMGB1, high mobility group box protein 1; HNE, 4-hydroxynonenal; JNK, c-jun N-terminal kinase; KEAP1, Kelch-like ECH-associated protein 1; LAMP, lysosomal-associated membrane protein; LC3, light chain 3; LOOH, lipid hydroperoxides; LPO, lipid peroxidation; MAP kinase, mitogen activated protein kinase; MCP-1, monocyte chemoattractant protein-1; MDA, malondialdehyde; MnSOD, manganese superoxide dismutase; MPT, mitochondrial permeability transition; mTORC1, mammalian target of rapamycin complex 1; NAC, *N*-acetylcysteine; NAPQI, *N*-acetyl-*p*-benzoquinone imine; NF-κB, nuclear factor κB; NQO1, NAD(P)H:quinone oxidoreductase 1; NRF2, nuclear factor erythroid 2-related factor 2; PUFAs, polyunsaturated fatty acids; ROS, reactive oxygen species; SMAC/DIABLO, second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low pI; TLR, toll like receptor; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; UGT, UDP-glucuronosyltransferases.

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Ferroptosis;
Autophagy;
NRF2;
Innate immunity

relevance. The purpose of this review is to provide a framework of the model where mechanistically sound and clinically relevant data can be obtained. The discussion provides insight into the injury mechanisms and how to study it including the critical roles of drug metabolism, mitochondrial dysfunction, necrotic cell death, autophagy and the sterile inflammatory response. In addition, the most frequently made mistakes when using this model are discussed. Thus, considering these recommendations when studying APAP hepatotoxicity will facilitate the discovery of more clinically relevant interventions.

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1. Introduction

Acetaminophen (APAP) is a widely used analgesic and anti-pyretic drug, which is considered safe at therapeutics doses[1,2]. However, because of its presence in numerous over the counter and prescription medications, especially in western countries, intentional and unintentional overdosing occurs quite frequently. Overdoses can cause dose-dependent hepatotoxicity and in some cases even liver failure[3]. In the US and the UK, 46% and 70%, respectively, of all acute liver failure cases are caused by APAP overdose[4,5]. Thus, APAP hepatotoxicity is a significant clinical problem. When a mouse model of APAP hepatotoxicity was developed and it was recognized that a reactive metabolite of APAP causes glutathione depletion[6], it allowed the rapid discovery of *N*-acetylcysteine (NAC) as antidote against an APAP overdose[7]. In fact, NAC is still the only clinically approved antidote more than 40 years later. Although NAC proved to be highly effective in preventing liver injury after an APAP overdose[8], the therapeutic efficacy is highest when administered within 8 h after APAP[9]. The quest for a drug that extends the therapeutic window of NAC led to extensive studies investigating the mechanisms of cell death and liver injury after APAP. In addition, countless interventions have been tested over the years, more recently especially in the realm of natural products[10−13]. Each of these studies ends with the conclusion that "Compound X" may be a promising new antidote against APAP toxicity. However, most, if not all, of these compounds will never make it into clinical development because of poor experimental design and wrong mechanistic conclusions. In this review we will discuss the reasons for these failures and provide some recommendations on how to use the APAP hepatotoxicity model in a way that generates relevant data, which can provide the basis for solid mechanistic understanding and assessment of the efficacy of an intervention under clinically realistic conditions. The review focuses on the various aspects of the injury mechanism; for recommendations on how to properly investigate the regeneration and repair phase, the reader is referred to expert reviews on this topic[14−16].

2. Most common mistakes made when using the APAP hepatotoxicity model

In order to improve the experimental design of many studies, it is first necessary to recognize the most common mistakes. First, all compounds are supposed to be used therapeutically to treat drug hepatotoxicity in patients. However, in most preclinical experiments the drug is administered as a pretreatment, *i.e.*, a single dose of the drug or daily pretreatment for up to 14 days. This raises serious concerns regarding the translation of these effects to a clinically relevant situation. Second, APAP hepatotoxicity is a time dependent event involving a number of different phases critical for the injury and recovery process (Table 1)[1,17−20]. However, the majority of all studies use a single, late time point. Then, as many different parameters as possible are measured, leading to conclusions of antioxidant, anti-apoptotic, and anti-

Table 1 Most common parameters to be measured across the temporal course of the APAP pathophysiology in the mouse model.

Phase	Parameter
Metabolism phase 0−3 h post APAP (depending on the dose)	- Cytochrome P450 activity (Liver) - NAPQI protein adducts (Liver or Plasma) - Glutathione depletion (Liver) - APAP metabolites (Liver or Plasma)
Early injury phase 2−6 h post APAP	- JNK activation and mitochondrial translocation (Liver) - Mitochondrial BAX translocation (Liver) - Mitochondrial superoxide formation (Liver) - Mitochondrial permeability transition (Liver) - Glutathione recovery (Liver) - ALT/AST activities (Plasma) - Area of necrosis (H&E) (Liver) - Nuclear DNA fragmentation (TUNEL) (Liver) - Nuclear DNA fragmentation (anti-histone ELISA) (Liver and Plasma)
Late injury−early recovery phase 12−24 h post APAP	- ALT/AST activities (Plasma) - Area of necrosis (H&E) (Liver) - Nuclear DNA fragmentation (TUNEL) and anti-histone ELISA (Liver and Plasma) - Innate immune response (PMN, Monocytes) (Liver) - PCNA (Liver)
Regeneration phase 24−96 h post APAP	- Resolution of necrotic area - PCNA and Ki67 expression - Cyclin D1 - Innate immune response (PMN, Monocytes) (Liver)

inflammatory properties of interventions and/or suggestions that the drug may promote autophagy or NRF2 activation[10−13]. However, it is rarely evaluated if any of the postulated mechanisms are actually the cause of the protection. Thus, the mechanistic conclusions are mostly questionable because it is unclear if the postulated mechanistic effects are the primary mode of action of the drug or just secondary effects and correlations. This problem seems to be caused mainly by the fact that investigators copy the poor experimental designs from previously published similar studies. Unfortunately, this leads to an ever-increasing number of published manuscripts despite the fact that they have limited relevance both in terms of evaluating the proposed therapeutic potential and the mechanistic understanding of drug action in this model.

3. General recommendations for investigating therapeutic efficacy and mechanisms of action

3.1. Animal models

3.1.1. Recommendations

To perform intervention studies that may be relevant for humans, it is important to choose the right animal model. Although the rat is an important preclinical animal species for drug development, APAP toxicity in the rat is limited. An APAP overdose triggers *N*-acetyl-*p*-benzoquinone imine (NAPQI) formation, GSH depletion and protein adduct formation but there is no evidence of relevant JNK activation, mitochondrial oxidant stress, DNA fragmentation and only very minor cell necrosis in the rat[21]. In contrast, the pathophysiology of severe liver injury induced by an APAP overdose in mice or mouse hepatocytes matches most aspects of the injury in human hepatocytes[22,23] and in patients[24−26] with one exception: the time course of the injury mechanism is intrinsically more delayed in humans or human hepatocytes compared to mice. However, there are a large number of different mouse strains, which considerably vary in their susceptibility to an APAP overdose[27]. Although most basic mechanisms of APAP toxicity are similar in various strains, there can be genetic differences that substantially modulate the injury pathways. An example is the most frequently used mouse strain to study mechanisms of APAP hepatotoxicity, C57BL/6, which actually has 2 substrains, 6N and 6J. C57BL/6N mice can be considered the wild type strain because C57BL/6J mice have a loss-of-function mutation of the nicotinamide nucleotide transhydrogenase (*Nnt*) gene[28]. This enzyme is located inside mitochondria and transfers electrons from NADH to $NADP^+$, and thus supports detoxification of reactive oxygen species by providing reducing equivalents for glutathione peroxidase. However, C57BL/6J mice have a lower mitochondrial oxidant stress and are less susceptible to APAP toxicity than the C57BL/6N strain[29]. The explanation for these unexpected findings is the fact that under conditions where the mitochondrial electron transport chain is inhibited, the enzyme works in the opposite direction[30]. Under stress, the enzyme transfers electrons from NADPH to NAD^+, which impairs the detoxification of reactive oxygen and through oversupply of NADH, promotes electron leak from the mitochondrial electron transport chain and thus, superoxide formation[30]. This variable susceptibility to APAP in different mouse strains has important implications when wild type and gene knock-out mice are from different backgrounds, even if the difference is in substrains of the background[31]. This is illustrated by the initially unrecognized problem when wild type and JNK2 knock-out mice from mismatched backgrounds were used in APAP studies, which led to early contradictory results from different laboratories around the importance of JNK in the pathophysiology of APAP toxicity[32−35]. Thus, it is critical to use littermate controls whenever possible and not just wild type mice from an assumed background strain, especially when mice are obtained from different vendors[31].

Another important issue is the higher susceptibility of male compared to female mice to an APAP overdose[36−38]. Although mice of both genders induce the transcription of the rate-limiting enzyme of glutathione synthesis, glutamate cysteine ligase (*Gcl*) after GSH depletion, the induction of *Gcl* and thus the rate of GSH synthesis is faster in female mice leading to lower toxicity[36]. Inhibition of GSH synthesis with buthionine sulfoxide in female mice increases their susceptibility to APAP to the level of male mice[37]. Thus, although the mechanism of APAP toxicity is similar in male and female mice, female require a higher dose of APAP than male to achieve the same degree of injury[38].

3.2. Drug treatment regimen

3.2.1. Recommendations

Any compound that may be considered for therapeutic use has to be tested as a co-treatment and at various times post APAP treatment using clinically relevant doses. In addition, any potential effect of the intervention on the different phases of the APAP-induced injury mechanism needs to be investigated to get an idea about which mechanism the drug is targeting. Once that is established, additional interventions need to be applied to establish the causality. Besides these general recommendations, specific aspects of investigating each injury phase or mechanism need to be considered as discussed in the following chapters.

3.3. Drug metabolism and cytochrome P450 enzymes

3.3.1. Background

APAP is predominantly metabolized by phase II reactions (glucuronidation, sulfation) but a small fraction (5%−10%) is converted to a reactive metabolite, NAPQI, by cytochrome P450 enzymes with CYP2E1 being the main enzyme[39]. First recognized by Mitchell and co-workers[40,41], it is now well established that this metabolic activation of APAP leads to extensive GSH depletion and then covalent binding of the reactive metabolite to proteins, which initiates the toxicity[19]. However, it is not the total protein binding in the cell that is critical for hepatotoxicity but the adduct formation on mitochondria[42,43], which triggers the early mitochondrial superoxide release[44] leading to all the subsequent signaling events characteristic of APAP-induced programmed necrosis[45,46]. This means that the entire mechanism of APAP-induced cell death depends on this CYP-mediated NAPQI formation and its reaction with GSH or protein sulfhydryl groups (Fig. 1). Thus, any interference with these reactions will lead to profound protection in this model *in vivo* or *in vitro* as shown with a potent CYP2E1 inhibitor[47] or with accelerated GSH re-synthesis[48,49].

3.3.2. Recommendations (in vivo experiments)

Any intervention used in the APAP model as a pretreatment or a co-treatment with APAP has to be evaluated for its effect on the initial metabolic activation. Importantly, this also includes the solvent as many compounds have to be solubilized in organic solvents such as dimethylsulfoxide (DMSO), which also is a potent inhibitor of P450 enzymes[50]. There are a number of assays

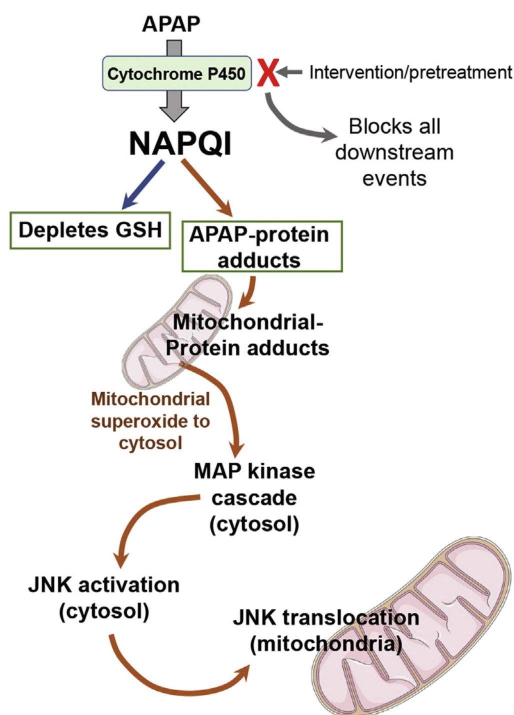

Figure 1 Acetaminophen metabolism and the initiation of the toxicity. APAP metabolism to the reactive intermediate NAPQI is a mandatory step for induction of liver injury: Though a minor metabolite under therapeutic APAP doses, excessive formation of the reactive metabolite NAPQI mediated by CYP450 is required for hepatotoxicity after an APAP overdose. While hepatic glutathione stores scavenge NAPQI, this causes depletion of hepatic GSH and subsequent formation of adducts on cellular proteins, especially on mitochondrial proteins. Initial mitochondrial protein adduct formation induces enhanced superoxide production towards the cytosol from the organelle, which then activates a MAP kinase cascade in the cytosol ultimately activating c-jun N-terminal kinase (JNK) by its phosphorylation. Cytosolic phospho-JNK then translocates to the mitochondria to ultimately induce mitochondrial dysfunction which contributes to hepatocyte necrosis. Since CYP450 catalyzed NAPQI formation is critical to initiation of this pathway, any intervention or pre-treatment which inhibits CYP450 would block all these downstream events. Though evaluation of hepatocyte necrosis under these conditions would show protection by the intervention, this will have no clinical relevance since this artifactual protection does not replicate the situation in humans, where the patients rarely take an APAP overdose and immediately present to the clinic for treatment. Hence, indices of APAP metabolism such as depletion of GSH or formation of protein adducts (green boxes) need to be evaluated with an intervention to confirm that APAP metabolism is not compromised. This figure includes templates from Servier Medical Art, which is licensed under a Creative Commons Attribution 3.0 generic license; https://smart.servier.com.

that can be used to evaluate the potential interference of a chemical in the metabolic activation of APAP. Although NAPQI cannot be measured directly, the initial exponential GSH depletion during the first 30 min after APAP[51], oxidative metabolites (APAP-GSH and its metabolites APAP-CYS and APAP-NAC) and protein adducts in the liver and plasma during the first 1−2 h in mice *in vivo*[47] or up to 6 h in cultured hepatocytes will give some reliable indication whether a drug will interfere with the most upstream events of the toxicity. Importantly, similar parameters can be measured in human hepatocytes[22] and in humans[26,52]. Although CYP2E1 mRNA and protein expression can be assessed, these parameters are only relevant if an intervention substantially modulates the protein expression at the time of APAP administration. However, protein expression levels do not allow insight into whether an intervention affects the enzyme activity. This can only be assessed *in vivo* with the parameters mentioned above or by a separate *in vitro* experiment where the dose-dependent effect of the drug on CYP enzyme activities in liver homogenate or microsomes is investigated using a CYP-specific substrate[47]. Alternatively, if the investigators want to make sure that there is no effect of the intervention on drug metabolism, the drug can be administered only after the metabolism of APAP is completed, which would be 90−120 min after a 300 mg/kg APAP dose in mice[53].

In addition to the direct effect of drugs on cytochrome P450 enzymes that can affect NAPQI formation and toxicity, indirect effects also need to be considered. Any modulation of the protein expression or the enzyme activities of the phase II enzymes can influence the amount of APAP that is metabolized through the P450 pathway and thus modulate toxicity. Furthermore, basal levels of GSH determine how much NAPQI is detoxified or will bind to proteins. Because drugs can protect or increase APAP toxicity by modulating hepatic GSH levels, it is important to determine the basal levels of hepatic GSH before APAP treatment. This is particularly important when the intervention involves pretreatment of compounds or extracts.

3.3.3. *Recommendations (in vitro experiments)*

The consideration of an intervention's effect on metabolic activation of APAP is also pertinent in *in vitro* studies regarding the cell types being chosen for analysis. It is critical to confirm that the hepatocytes being used *in vitro* are capable of generating NAPQI from APAP to replicate cell death mechanisms occurring *in vivo*. Thus, primary mouse or human hepatocytes need to be exposed to APAP within a few hours after allowing for attachment on tissue culture plates, since prolonged 2D culture *in vitro* can result in loss of metabolic capability in these cells. Although 3D culture techniques can maintain metabolic capacity in hepatocytes much longer than regularly cultured hepatocytes[54], the additional effort is generally not needed for the acute injury experiments with APAP. However, concerns with expression of drug metabolizing enzymes are especially true when immortalized cell lines are being used to study APAP toxicity, since many hepatocyte cell lines lack the metabolic capability to generate sufficient NAPQI from APAP. One exception is HepaRG cells[55], which have been shown to recapitulate almost all mechanistic indices of APAP pathophysiology *in vivo*[23], except JNK activation[22]. This contrasts with other cell lines such as HepG2 cells which lack most drug metabolizing enzymes[56,57] and thus cannot metabolize APAP to NAPQI and are hence not a good model to test the effect of interventions in APAP pathophysiology[23]. Therefore, the use of immortalized cell lines other than HepaRG cells should be avoided in APAP studies.

In summary, assessing the effect of a single compound or a mixture from an extract on the metabolic activation of APAP is absolutely critical for the interpretation of the results and the proper mechanistic conclusions when an intervention is used as a pre-treatment or during the metabolism phase. This evaluation can only be avoided when the treatment is started after drug metabolism, which is a time window that is dose-dependent and can be

affected by the experimental model and the species and strain that is being used. Not considering an effect on drug metabolism is among the most frequently made mistakes that can lead to severe misinterpretation of the mechanism of protection of the intervention being studied.

3.4. *Mitochondrial dysfunction and oxidant stress*

3.4.1. *Background*

As discussed, mitochondrial proteins are targets for adducts formation by NAPQI, which triggers a mitochondrial oxidant stress[48,51,58,59]. However, there are two distinct phases of enhanced mitochondrial free radical generation. The initial phase triggered by protein adducts involves the early release of superoxide radicals from mitochondrial respiratory complex III towards the cytosol (Fig. 1), which occurs without compromising mitochondrial function[44], and induces mitogen-activated protein (MAP) kinase signaling within the cytosol activating c-jun N-terminal kinase (JNK)[60]. It is JNK translocation to mitochondria[61] which initiates amplification of the oxidant stress and ultimately compromises mitochondrial respiration (Fig. 2)[62,63]. Phosphorylated JNK binds to the mitochondrial outer membrane scaffold protein Sab[64] and inhibits mitochondrial electron transport through a Src mediated process[65]. Elevated mitochondrial free radical production in this amplification phase then seems to be through respiratory complex I, which is a predominant site of ROS formation[66–68]. Severity of APAP-induced liver injury was correlated to complex I activity[69], and treatment with metformin, which is a complex I inhibitor, prevented oxidative stress and liver injury in mice as well as human HepaRG cells[69]. Elevated superoxide production within mitochondria has been documented in cultured mouse hepatocytes[48,58,70] and the human HepaRG cell line exposed to APAP[23] and also *in vivo* in liver mitochondria from APAP treated mice[69].

Mitochondria have robust antioxidant defenses such as manganese superoxide dismutase (MnSOD) to counteract such elevations in matrix superoxide production, which is evident in the increased susceptibility of mice with partial MnSOD deficiency to APAP-induced liver injury[71,72]. The resulting dismutation product, hydrogen peroxide, can also be directly scavenged by GSH or enzymes such as glutathione peroxidase or catalase[51,73]. So why do we have mitochondrial dysfunction after JNK translocation despite these antioxidant systems? This is possibly due to active compromise of electron transport by JNK-mediated signaling within the mitochondria, coupled with formation of peroxynitrite[60]. This highly reactive nitrogen species is formed by reaction of superoxide with nitric oxide within the mitochondria and a point to note is that an examination of the time course of peroxynitrite formation reveals that it is only evident in mitochondria after JNK translocation[59,74], with no peroxynitrite detected during the very early superoxide increase prior to JNK activation in the cytosol[44]. The elevation in mitochondrial peroxynitrite also nitrates and inhibits MnSOD activity[75], which in turn compromises superoxide scavenging, and also modifies mitochondrial DNA[76]. In addition, peroxynitrite effectively reacts with GSH and thus ensures a low level of this antioxidant in mitochondria[49]. The importance of this elevated mitochondrial superoxide and peroxynitrite formation to APAP hepatotoxicity is illustrated by the efficacy of interventions which directly target these oxidants. Treatment with the SOD mimetic Mito-TEMPO protects against APAP-induced mitochondrial oxidant stress and hepatocyte necrosis *in vivo*[77]. Moreover, enhanced GSH re-synthesis induced by providing cysteine[49,78,79] or increased expression of rate-limiting enzymes, *e.g.*, GCLC[36], results in improved scavenging of peroxynitrite and profound protection.

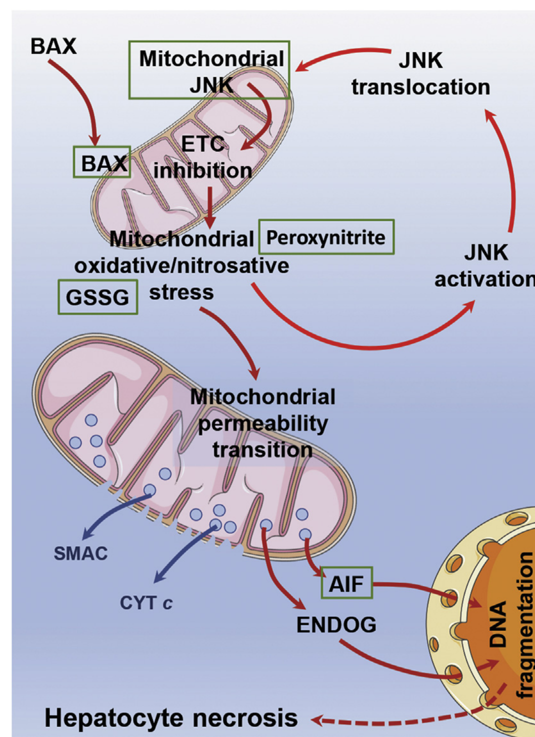

Figure 2 The propagation phase of acetaminophen-induced cell death. Mitochondrial dysfunction is central to APAP pathophysiology: Activation of JNK in the cytosol and its translocation to the mitochondria initiates a vicious cycle of mitochondrial distress, where inhibition of mitochondrial electron transport enhances reactive oxygen generation and formation of peroxynitrite, which cause mitochondrial oxidative and nitrosative stress. This in turn further amplifies the cytosolic MAP kinase cascade activating JNK and maintaining its translocation to the mitochondria. Persistent mitochondrial oxidative and nitrosative stress evidenced by increase in glutathione disulfide (GSSG) and peroxynitrite, coupled with BAX translocation to the mitochondria ultimately induce the mitochondrial permeability transition (MPT). This results in release of mitochondrial proteins such as SMAC, cytochrome *c*, apoptosis inducing factor (AIF) and endonuclease G (ENDOG) into the cytosol. AIF and ENDOG translocate to the nucleus to induce DNA fragmentation and ultimately hepatocyte necrosis. These well-defined steps in APAP pathophysiology allow their examination by evaluating JNK and BAX as well as glutathione disulfide and nitrotyrosine protein adducts (as marker of peroxynitrite formation) in mitochondrial fractions (green boxes) which would provide mechanistically relevant information rather than their analysis in the whole liver. Likewise, examination of mitochondrial proteins such as AIF in the cytosolic fraction provide information on activation of the MPT *in vivo*. This figure includes templates from Servier Medical Art, which is licensed under a Creative Commons Attribution 3.0 generic license; https://smart.servier.com.

The elevation in mitochondrial peroxynitrite formation with the accompanying inhibition of anti-oxidant systems and nitration of mitochondrial components induces alterations in mitochondrial membrane potential and morphology, which, though reversible at the early stages[80], ultimately results in irreversible collapse of the mitochondrial membrane potential and induction

of the mitochondrial permeability transition (MPT) (Fig. 2)[81−83]. Unlike the earlier morphological changes driven by membrane potential[80], the later mitochondrial fission is mediated by alterations in classical proteins involved in mitochondrial dynamics such as Mitofusin 2 and Drp 1[80,84]. The importance of MPT induction in APAP pathophysiology is illustrated by protection afforded by inhibitors targeting cyclophilin D[83], which is a well characterized component of the MPT[85]. However, the transient nature of this protection[81], and the APAP dose response seen in cyclophilin D-deficient mice, with mice protected against a mild APAP overdose[83], but not against a severe one[86], indicates that induction of the MPT is probably dependent on the magnitude of APAP-induced upstream signaling. This is also supported by the fact that while mice treated with lower APAP overdose of 150 mg/kg showed a transient induction of MPT, those receiving a higher 300 mg/kg dose had an irreversible MPT[87]. The final consequence of the MPT is the release of mitochondrial proteins such as apoptosis-inducing factor (AIF) and endonuclease G (ENDOG) into the cytosol and subsequent translocation to the nucleus (Fig. 2)[88,89]. While ENDOG cleaves nuclear DNA causing its fragmentation[90], AIF condenses chromatin and induces DNA fragmentation[91]. Mice partially deficient in AIF have been shown to be protected against APAP hepatotoxicity[92], illustrating the importance of these mitochondrial proteins in APAP pathophysiology.

3.4.2. Recommendations

Given the central role of mitochondrial dysfunction in APAP-induced cell death, it is critical to specifically assess mitochondrial function. Unfortunately, most studies neglect to evaluate the above-mentioned, well-defined pathways of the APAP pathophysiology and generally omit analysis of mitochondrial function when using the model[93,94]. The mere focus on measurement of BAX protein levels rather than its mitochondrial translocation, which is the pathophysiological relevant feature, is another common issue[93,95]. Even studies which purport to evaluate JNK activation merely examine changes in the whole liver rather than a more relevant examination of its mitochondrial translocation[95]. To gain relevant insight into the protective role of an intervention in APAP pathophysiology, it is imperative that the well characterized pathways of APAP-induced cell signaling, from protein adduct formation to mitochondrial dysfunction (Fig. 2), be systematically studied in a step wise manner. For *in vivo* studies, once it has been determined that the intervention does not interfere with APAP metabolism and induces early GSH depletion as in animals treated with APAP alone, the next step would be subcellular fractionation to separate mitochondrial and cytosolic fractions to evaluate JNK translocation to mitochondria and assess mitochondrial GSSG levels as indicator of a mitochondrial oxidant stress. It has to be kept in mind that JNK activation in the cytosol and its mitochondrial translocation are early events (Table 1), which need to be measured between 1 h and maximally up to 6 h after APAP administration in mouse livers *in vivo* or mouse hepatocytes[44,61,74] and 6−16 h after APAP in primary human hepatocytes[22]. Furthermore, hepatic GSH levels reflect mainly NAPQI formation during the first 1 h (up to 3 h after high doses) after APAP in mice but after that indicate recovery, which may vary dependent on the degree of injury[96]. GSSG levels are difficult to measure during maximal GSH depletion (0.5−3 h after APAP treatment in mice) but can be measured more accurately during the recovery phase[51,59]. At baseline, GSSG levels represent 0.5% of the total GSH in the liver; during APAP toxicity, GSSG levels can increase to 2%−5% between 6 and 24 h after APAP, reflecting a significant oxidant stress[51,59]. Importantly, as the oxidant stress is located predominantly in the mitochondria, GSSG levels measured in mitochondria are even higher (up to 20%)[51,59,79]. However, accurate measurement of hepatic GSSG levels requires snap-freezing the tissue in liquid nitrogen, strict storage at −80 °C, and the use of diethyl maleate as a GSH-trapping agent when homogenizing the liver samples[97].

These experiments could be complemented by *in vitro* studies in primary hepatocytes, where effects of treatment on APAP-induced loss of mitochondrial membrane potential can also be tested (JC-1 assay). Peroxynitrite formation can be examined by immunostaining for nitrotyrosine[49] and induction of the MPT can then be evaluated *in vivo* by examination of release of mitochondrial proteins such as AIF, ENDOG and SMAC/DIABLO into the cytosol and translocation to the nucleus[88,98].

3.5. Lipid peroxidation and ferroptosis

3.5.1. Background

Ferroptosis is a term which was first used to describe the form of cell death induced by compounds such as erastin, which are selectively lethal to RAS mutant cells[99]. Ferroptosis is triggered by the iron dependent accumulation of lipid peroxides, which are formed when polyunsaturated fatty acids (PUFAs) present in phospholipids react with reactive oxygen species to generate lipid hydroperoxides (LOOH). Iron facilitates this process by reacting with LOOH in the Fenton reaction to generate highly reactive lipid free radicals. These lipid free radicals go on to react with other PUFAs propagating a chain reaction which, if left unchecked, leads to accumulation of lipid peroxides[100]. The accumulation of these lipid peroxides in membranes may then cause ferroptotic cell death due to perturbation of membrane integrity and permeability, oxidation of membrane bound proteins, as well as generation of cytotoxic peroxidation by-products such as malondialdehyde (MDA) and 4-hydroxynonenal (HNE)[101,102]. Under normal physiological conditions, this process is prevented by several defense mechanisms, chief of which is the glutathione peroxidase 4-GSH system. GPX4 is a selenoperoxidase which detoxifies lipid peroxides by reducing them to their alcohol derivative[103]. Thus, one way to induce ferroptosis is by inhibition of GPX4 activity, either directly (as is the case with RSL3) or *via* prevention of glutathione synthesis such as with erastin[104]. Another endogenous defense against ferroptosis is *via* the ferroptosis suppressing protein 1 (FSP1) which helps to prevent lipid peroxidation (LPO) by generating ubiquinol (coenzyme Q10) and vitamin E, a powerful chain-breaking lipophilic antioxidant[105].

Despite ferroptosis being a relatively new term, the role of LPO in APAP hepatotoxicity has been investigated extensively for decades[106−109]. The key finding of these previous studies was that vitamin E-deficiency and high levels of PUFAs in cell membranes due to feeding a diet high in soybean oil, renders animals highly susceptible to LPO and thus, LPO becomes the main mechanism of liver injury with massive increases of LPO parameters[110,111]. However, under normal circumstances when animals are fed a regular diet, ethane exhalation and other LPO markers such as MDA only increased 2−3-fold[112−114] or did not increase at all[115]. When compared to the orders of magnitude increase observed in known models of lipid peroxidation-induced cell death[115,116], these comparatively small increases in LPO markers suggest that LPO does not occur at a high enough level to directly cause cell death in APAP hepatotoxicity.

3.5.2. Recommendations

When measuring LPO markers in APAP-induced liver injury, which includes mainly MDA and HNE, quantitative aspects need to be kept in mind. Does it make sense that doubling of MDA levels is responsible for massive liver injury as observed in APAP overdose mice or patients? In addition, the use of ferroptosis inhibitors has been adopted as a tool to study the role of ferroptosis in the model. Increasing membrane vitamin E levels by 7-fold has been shown to have protective effects in LPO-induced liver injury models[115]. However, whether vitamin E is protective in APAP-induced liver injury remains highly disputed as conflicting studies have been published[114,115,117]. Pharmacological iron chelation has also been evaluated as a possible protective intervention in APAP liver injury. Iron chelators, *e.g*., deferoxamine and starch desferal, have been shown to be partially protective against APAP-induced liver injury[118−121]. However, these results must be interpreted with caution. Iron is involved in numerous cellular processes and the chelation of redox active iron could potentially cause several off-target effects which could be responsible for the observed protection of iron chelators. To this point, iron has been shown to promote induction of the mitochondrial permeability transition, a step which is critical in the progression of APAP induced liver injury[120,122]. In addition, nitration of proteins such as MnSOD by peroxynitrite is essential for the amplification of oxidant stress and mitochondrial dysfunction which precedes APAP mediated cell death[123]. Iron has also been shown to catalyze this process[124], and deferoxamine has been shown to inhibit peroxynitrite-mediated protein nitration[125], providing another possible explanation for the protection by iron chelators. Therefore, more care needs to be taken to avoid using off-target effects of iron chelators as evidence of significant ferroptosis in APAP hepatotoxicity. However, iron chelators are not the only ferroptosis inhibitors with relevant off-target effects. A recent study claimed that (+)-clausenamide protected against APAP induced liver injury by inhibiting ferroptosis[126]. However, it is shown in the same paper that the compound upregulates NRF2, which is known to bind to the antioxidant response element (ARE) which induces endogenous antioxidant defense mechanisms[127]. Ferrostatin-1, another ferroptosis inhibitor, which acts as a lipophilic radical trapping antioxidant[128], has also been reported to protect against liver injury in APAP overdose[117]. Interestingly ferrostatin-1 only protected as a pretreatment and effects on APAP metabolism were not evaluated, highlighting some of the pitfalls already discussed. In addition, the authors also showed an unexplained uncharacteristically high level of injury following a modest overdose of 200 mg/kg APAP as early as 3 h[117].

In summary, LPO is quantitatively insufficient to cause APAP-induced cell death unless defense mechanisms (vitamin E) are severely depleted and the targets of peroxidation, polyunsaturated fatty acids, are significantly elevated through diet. Thus, ferroptosis is not a relevant mode of cell death in APAP hepatotoxicity under normal conditions[45,46,129]. However, there is increasing evidence for involvement of iron in the pathophysiology, suggesting additional mechanisms than catalyzing the classical Fenton reaction.

3.6. Apoptosis

3.6.1. Background

Apoptosis is a form of programmed cell death generally designed to remove cells no longer needed during development or eliminate cells in the tissue that have sustained some damage and reached the end of their natural lifespan. Importantly, it generally affects individual cells, which show characteristic morphological changes including cell shrinkage, chromatin condensation and margination but still intact cell organelles[130]. Eventually the cell breaks up into apoptotic bodies, which are removed by macrophages or neighboring cells in a process termed efferocytosis[131,132]. The signaling events responsible for extrinsic and intrinsic apoptotic cell death are well studied and described in detail in many reviews[46,130,133]. Importantly, all apoptotic signaling events can be executed by altering interactions and intracellular compartmentalization of existing proteins, *i.e*., it does not require transcriptional activation of genes and new protein synthesis.

3.6.2. Pitfalls and recommendations

Although it was generally assumed that APAP overdose causes necrotic cell death, early suggestions of APAP-induced apoptosis were based on misinterpretations of nuclear DNA fragmentation as being specific for apoptosis and questionable morphological changes[134]. However, more detailed quantitative morphological assessment of cell death showed overwhelming necrosis as indicated by cell and organelle swelling, karyorrhexis and karyolysis, and release of cell contents with inflammation[135]. In addition, there was no caspase activation[136,137] and potent pancaspase inhibitors did not protect[50,117,135,136,138]. Interestingly, DNA fragmentation showed small (multiples of 180−185 base-pairs in length) and larger fragments[76,139]. However, this is not caused by caspase-activated DNase (CAD) but mitochondrial-derived endonucleases such as AIF and ENDOG[88]. In addition, BAX and truncated BID translocate to mitochondria[140,141] and accelerate APAP-induced cell death by DNA fragmentation[98,142]. Furthermore, APAP-induced necrotic cell death also involves cytochrome *c* and SMAC/DIABLO release from mitochondria but this does not trigger caspase activation[98]. Taken together, necrotic cell death in response to APAP overdose shows some overlap with apoptosis signaling but key events of apoptosis are absent (Fig. 2). This is not only relevant for mice or mouse hepatocytes but also for human hepatocytes and patients exposed to an APAP overdose[22,23].

More recent studies where it is claimed APAP causes apoptosis, base these conclusions mainly on increased expression levels of mRNA or proteins of pro-apoptotic genes such as BAX, and caspase-3 or declining expression of anti-apoptotic genes like *Bcl-2* (reviewed in Refs.143,144). In addition, TUNEL staining is being used, although it is known for many years that the DNA strand breaks detected by this assay are not specific for apoptosis[145]. However, the key to reliably detecting apoptosis are the characteristic morphological changes and caspase activation that is quantitatively proportional to the degree of apoptosis. Ultimately, if apoptosis is relevant for any pathophysiology, pancaspase inhibitors are highly effective in eliminating the injury. Unfortunately, when the proper solvent controls are employed, nobody has ever shown a beneficial effect of caspase inhibition against APAP hepatotoxicity. Therefore, apoptotic cell death does not contribute in a meaningful way to APAP-induced liver injury and therefore, measuring parameters of apoptosis is of limited use in this model.

3.7. Inflammation—injury and recovery

3.7.1. Background

The mechanism of APAP hepatotoxicity is associated with a sterile inflammatory response due to the massive necrotic cell

death that occurs during the injury phase (0−12 h after APAP in mice)[146]. Damage associated molecular patterns (DAMPs) are released during necrosis including high molecular group box 1 (HMGB1) protein, mitochondrial and nuclear DNA, ATP, uric acid, and many others (Fig. 3)[147]. HMGB1 can bind to toll like receptor 4 (TLR4) on Kupffer cells and activate the transcription factor nuclear factor κB (NF-κB) pathway to release tumor necrosis factor (TNF-α)[148]. Extracellular ATP signals *via* the purinergic receptor P2X7 on Kupffer cells and triggers plasma membrane pore formation and release of potassium that activates Nek-7 and leads to assembly of the NALP3 inflammasome and activation of pro-caspase 1[149]. Active caspase 1 cleaves pro-IL-1β or pro-IL-18 to produce the active cytokines IL-1β and IL-18. Mitochondrial DNA and nuclear DNA fragments can transcriptionally activate the formation of pro-IL-1β through the TLR9 receptor[150,151]. As pro-inflammatory cytokines, both TNF-α and IL-1β can facilitate immune cell activation and hepatic infiltration, especially neutrophils as the first responder[152]. TNF-α can also induce formation of MCP-1[153], which recruits bone marrow-derived monocytes into the liver after an APAP overdose[154]. The main purpose of a sterile inflammatory response in the liver is to remove the dead cells and promote tissue repair (Fig. 3). Under certain conditions, neutrophils, the resident macrophages (Kupffer cells) and the newly recruited monocytes/macrophages can also aggravate liver injury in models of hepatic ischemia-reperfusion injury or obstructive cholestasis[155,156].

3.7.2. *Pitfalls and recommendations*

The preponderance of experimental evidence in both animals and humans after APAP hepatotoxicity suggest that the inflammatory cells promote regeneration and do not cause additional injury[18,147]. In fact, deficiency of NADPH oxidase (NOX2) activity in all inflammatory cells, which prevents any immune cell-mediated oxidant stress, does not attenuate the APAP-induced oxidant stress or the injury[157,159], which makes it virtually impossible that any of these phagocytes contribute to the injury process. In contrast, it is quite clear that inactivating these inflammatory cells or preventing them from being recruited into the liver attenuates regeneration and recovery of the tissue[154,160−163]. Thus, the frequent claim that drugs protect in the APAP model by their anti-inflammatory action must be questioned (Fig. 3).

In most cases when potential interventions against APAP toxicity are being tested, parameters of inflammation are measured, which include formation of various cytokines (mRNA and protein levels) such as TNF-α and IL-1β, chemokines, NF-κB activation and potentially recruitment of neutrophils and monocytes into the liver[10−13]. The results are generally that compared to an untreated control, all inflammatory parameters are increased in the APAP-treated animal and these parameters are attenuated in the drug-treated group in parallel to reduced injury. These types of results do not justify the conclusion that the drug has anti-inflammatory effects. First, if the drug affects the injury mechanism in hepatocytes (inhibits P450 or JNK activation, prevents mitochondrial oxidant stress and dysfunction, etc.), the reduced injury will result in less DAMPs release and less inflammation; in other words, the reduced inflammatory response is a consequence of the reduced injury mechanisms and is not caused by a direct effect on cytokine/chemokine formation and inflammatory cell recruitment (Fig. 3). Second, if the reduced inflammation and attenuated liver injury show a positive correlation, this does not prove causality, *i.e.*, that the lower inflammatory response was the cause of the protection. In fact, given that extensive mechanistic

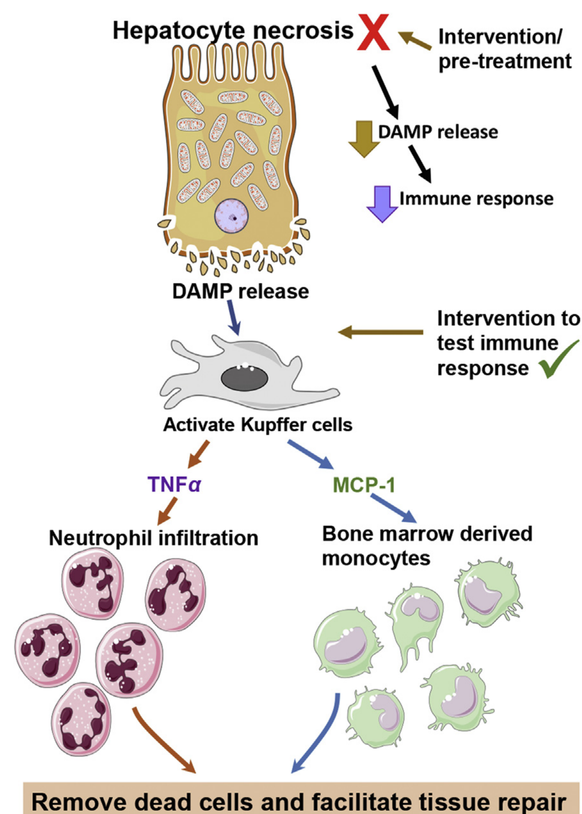

Figure 3 The sterile inflammatory response after acetaminophen-induced cell death. The innate immune response facilitates liver recovery and repair after APAP-induced injury: Hepatocyte necrosis causes release of cell components which function as damage associated molecular patterns (DAMPs) which induce Kupffer cells to release cytokines such as TNF-α and MCP-1, which results in infiltration of neutrophils and bone marrow derived monocytes into the liver, which facilitate removal of dead cells and liver regeneration and recovery. Interventions or pre-treatment strategies which prevent hepatocyte necrosis upstream would thus prevent this immune response which could be falsely interpreted as an anti-inflammatory response. To confirm that the intervention modulates the innate immune response, it should be administered in a delayed fashion, subsequent to hepatocyte necrosis. This figure includes templates from Servier Medical Art, which is licensed under a Creative Commons Attribution 3.0 generic license; https://smart.servier.com.

studies into the role of inflammation in this model[18,146,147] and in patients[158,164] do not support a contribution of inflammatory cells in the pathophysiology, it is highly unlikely that most drugs have a direct anti-inflammatory effect. However, if these aspects need to be investigated it would be critical to use the intervention only towards the end of the injury phase (6−12 h after APAP treatment in mice) to avoid interference with the injury mechanism. This issue could be important when a pro-inflammatory mediator, instead of affecting inflammatory cell recruitment, impacts intracellular mechanisms of cell death in hepatocytes, *e.g.*, modulates peroxynitrite formation through induction of nitric oxide synthase[165]. In this respect, when using gene-deficient mice or when interventions are employed such as acute gene knock-down experiments or antibody-mediated depletion of inflammatory cells, *i.e.*, the manipulation occurs before APAP treatment, it is important to consider adaptive mechanisms leading to off-target effects.

An example is the intracellular preconditioning effect that occurs when animals are pretreated with neutropenia-inducing antibodies for 24 h[166]. As a result, the protection against APAP toxicity in these neutropenic animals is misinterpreted as evidence for a neutrophil-mediated injury[167] although with appropriate design it is obvious that the effect is independent of neutrophils[159].

In summary, inflammation is a common response to the extensive necrotic cell death after an APAP overdose in animals and in patients. However, this sterile inflammation is critical for the repair of the injury, but inflammatory cells are not directly involved in the injury mechanism. A reduction in inflammation parameters after a drug intervention with reduced liver injury does not justify the conclusion that there is a causal relationship. If it is hypothesized that a drug has direct anti-inflammatory effects, this needs to be tested using an appropriate experimental design. In this respect, it needs to be considered that an inflammatory mediator may affect intracellular signaling mechanisms of cell death.

3.8. Autophagy

3.8.1. Background

Autophagy (or macroautophagy) is a process that degrades cellular components and damaged organelles to maintain cellular homeostasis under physiological or chemical stresses as a survival mechanism. Autophagy is initiated with the activation of different autophagy-related genes (Atg) and proteins that are critical for autophagy regulation[168,169]. Autophagy starts with the formation of a double-membrane structure termed phagophore, followed by a gradual elongation of the structure resulting in a double-membrane vesicle that surrounds the cargo (autophagosome). The autophagosome membrane fuses with the lysosomal membrane forming an autolysosome, and the cargo present is degraded by lysosomal enzymes[168,169].

The molecular mechanisms of autophagy regulation under different physiological and pathophysiological conditions have been studied extensively[169−172]. When nutrients are sufficient, mTORC1 is activated and inhibits the Unc-51-like kinase 1 complex (ULK1, ATG1, ATG13, and FIP200). In contrast, during starvation and/or energy-deficient conditions, mTORC1 is inhibited and AMPK is activated, which in turn activate the ULK1 complex, initiating the phagophore formation by activation of beclin-1 and the autophagy-promoting class III phosphatidylinositol-3-kinase (Ptdlns3K) complex. Activation of Ptdlns3K complex activates recruitment of other ATG proteins to initiate the elongation process where microtubule-associated protein light chain 3 (LC3) is implicated. First, LC3 is processed by ATG4B exposing its C-terminal glycine to form initially LC3-I, which is then activated sequentially by ATG7 (E1-like), ATG3 (E2-like) and ATG5-ATG12-ATG16 (E3-like) to conjugate with phosphatidylethanolamine to form the membrane-associated LC3-II form. ATG proteins such as ATG9 as well as VMP1 and TMEM41B participate in the elongation and vesicle closing. Once the autophagosome is formed, it fuses with a lysosome leading to cargo degradation by lysosomal hydrolases. Lysosomal-associated membrane protein 2 (LAMP-2) and components of the SNARE family proteins, such as VAMP7, VAMP8, VAMP9, and STX17 are implicated in the fusion of autophagosomes with lysosomes[169−172].

APAP-induced liver injury is characterized by the formation of APAP protein adducts, an increase in reactive oxygen species, mitochondrial dysfunction, and mitochondrial damage[17,19]. Removal of damaged mitochondria and protein adducts through autophagy is an important protective mechanism against APAP-induced liver injury[173,174]. APAP treatment significantly suppressed the activity of two downstream phosphorylation targets of mTOR in a dose-dependent manner[173] suggesting that an APAP overdose induces autophagy, which attempts to reduce hepatocyte necrosis by inhibition of mTOR and activation of AMPK. Autophagic removal of damaged mitochondria is mediated by a selective autophagy process termed mitophagy[175]. Mitophagy is accomplished by PARKIN-dependent and -independent pathways. In damaged and depolarized mitochondria, PINK1 senses the damage and is stabilized on the outer mitochondrial membrane where it phosphorylates PARKIN, which through its E3 ligase activity ubiquitinates proteins on the outer mitochondrial membrane. This can recruit soluble autophagy receptors such as P62 to the mitochondria. Next, P62 is further recruited to LC3 positive autophagosomes to initiate mitophagy[175,176]. In contrast, the PARKIN-independent pathway involves many autophagy receptors that are also mitochondrial resident receptors, such as BNIP3, NIX, and FUNDC1. These receptor proteins further recruit autophagosomes to mitochondria through direct interaction with LC3. Lipids also can bind LC3 and act as autophagy receptors. Cardiolipin, a mitochondrial phospholipid, in normal conditions is located on the inner mitochondrial membrane, when the membrane is depolarized after mitochondrial damage, cardiolipin is translocated from inner membrane to the outer mitochondrial membrane and directly interact with LC3 to trigger mitophagy[177].

It has been demonstrated that APAP administration increases PARKIN translocation to the outer mitochondrial membrane promoting mitophagy[178]. Administration of rapamycin to induce autophagy attenuates APAP-induced liver injury in mice, whereas autophagy inhibition by administration of chloroquine exacerbates APAP-induced hepatotoxicity[173] indicating the importance of autophagy for the pathophysiology. Acute knockdown of *PARKIN* using adenovirus vectors impaired autophagy and exacerbated APAP toxicity[178]. However, PARKIN knockout mice were partially protected suggesting that under chronic conditions APAP-induced autophagy can be PARKIN-independent and off-target effects can affect the injury process[178]. Similar observations were made with ATG5 knockout mice, which due to the chronic stress and accumulation of autophagy substrate protein P62 caused by inhibited autophagy, resulting in the persistent NRF2 activation as adaptive mechanisms that protected against APAP toxicity[179]. More recently it was shown that only PARKIN/PINK1 double knockout mice but not mice with the individual genes deleted, showed elevated liver injury compared to wildtype animals after APAP overdose[180]. In contrast, chronic overexpression of augmenter of liver regeneration (Hepatopoietin) with lentivirus transfection increased autophagy as indicted by the elevated number of autophagosomes, the conversion of LC3 I to LC3 II, and the degradation of P62, and attenuated APAP-induced liver injury by 50% consistent with the protective effect of autophagy activation in this model[181].

Mitochondrial dysfunction is central to APAP-induced cell death and removing these damaged mitochondria by mitophagy is beneficial[173]; however, there are a substantial number of extra-mitochondrial proteins that are also adducted by NAPQI. These protein adducts are also effectively removed by autophagy[174]. It is thought that mainly the adducts on mitochondrial proteins are critical for the initiation of the injury after an acute overdose[44] and thus removal of cytosolic protein adducts may be of limited

relevance. However, during chronic treatment with even therapeutic doses of APAP, the removal of these cytosolic protein adducts becomes important as any impairment of autophagy leads to accumulation of these adducts and liver injury even in the absence of mitochondrial protein adducts[182].

3.8.2. Pitfalls and recommendations

An increasing number of studies claim that pharmacological interventions protect in the APAP hepatotoxicity model due to activation of autophagy[10−13]. However, there are some concerns regarding these mechanistic conclusions. First, in order to properly assess the role of autophagy, appropriate methodology to measure autophagy and autophagic flux needs to be employed[183]. Second, it is essential to investigate whether the changes in autophagic flux in the drug-treated animals are actually the cause and not just a consequence of the protection through other mechanisms. Third, it has to be kept in mind that after an acute single overdose, autophagy can only modulate cell death in the peripheral areas of necrosis because in this zone the cellular stress is more moderate and delayed, allowing adaptive removal of damaged mitochondria to have an impact on cell survival[184]. Hence autophagy has a much more profound impact on APAP-induced liver injury after multiple, lower overdoses than after a single severe overdose[182]. Fourth, the standard model of APAP hepatotoxicity, *i.e.*, treating overnight fasted mice with a dose of 300 mg/kg APAP, is not the best approach when studying the effects of autophagy because starvation will activate autophagy. Thus, it is more appropriate to use fed mice with a higher dose of APAP (500 mg/kg).

3.9. NRF2 activation

3.9.1. Background

The KEAP1−NRF2 complex serves as a molecular sensor for the redox status within a cell[185]. Generally, during basal conditions nuclear factor erythroid 2-related factor 2 (NRF2) is targeted for ubiquitination which is mediated through Kelch-like ECH-associated protein 1 (KEAP1) and the Cullin 3 ubiquitin E3 ligase complex[186]. However, KEAP1 cysteine thiols will react with electrophilic compounds (*e.g.*, NAPQI) leading to the release of NRF2 by KEAP1[187], whereby NRF2 can translocate to the nucleus and activate antioxidant defense protocols[188]. Importantly, induction of critical genes involved in hepatic GSH resynthesis (*Gclc* and *Gclm*) following APAP toxicity[36,189] can mitigate injury as GSH can directly sequester NAPQI and detoxify reactive oxygen and peroxynitrite. Other canonical genes regulated by NRF2 include NAD(P)H:quinone oxidoreductase 1 (NQO1), glutathione peroxidase, and the conjugation enzymes, UDP-glucuronosyltransferases (UGT) and glutathione-*S*-transferases (GST)[190]. Collectively, these enzymes can detoxify NAPQI leading to a reduction in injury[191]. The observation that *Nrf2* knockout mice had greatly increased injury following APAP[192], and the identification of key detoxification enzymes under transcriptional control of NRF2 began an effort to identify 'NRF2 activators'[188]. Many pharmacological therapeutics and herbal remedies do enhance activation of NRF2 and protect against APAP toxicity[193−195], although it remains unclear if activation of the NRF2 transcription program is the only mechanism of protection[195,196]. This interpretation is further confounded because NRF2 is activated by APAP exposure alone[197].

3.9.2. Recommendations

Numerous recent studies have suggested NRF2 activation as the putative mechanism of protection based on increases in NRF2 protein levels or *Nrf2* gene expression levels. To properly assess NRF2 activation, it is necessary to separate the nuclear and cytosolic compartments to demonstrate that there is an increase in nuclear NRF2. Furthermore, NRF2 target genes, such as *Nqo1*, *Gclc*, *Gclm*, *Gsta1/a3*, and *Ugts* should be evaluated. Additionally, hepatic GSH levels can be measured as a functional readout for NRF2 activation. It is possible that much of the benefit derived from enhanced NRF2 activation is either caused by increased baseline levels of GSH or stems from accelerated resynthesis of hepatic GSH. Enhanced recovery of mitochondrial GSH levels is correlated with a reduction in liver injury following APAP treatment[78]. To mechanistically explore if NRF2 activation is mediating protection through GSH, BSO can be used to inhibit GCL (a heterodimeric enzyme consisting of the protein subunits encoded by *Gclc* and *Gclm*), which will effectively prevent GSH synthesis. BSO can be administered prior or subsequent to APAP overdose and has been found to be effective at doses ranging from 2 to 7 mmol/kg (dissolved in 0.9% saline)[197,198]. In addition, use of NRF2-deficient mice can give insight whether modulation of this transcription factor by the intervention is the cause of the protection. Taken together, these strategies establish *bona fide* NRF2 activation and a potential direct mechanism conferring protection. Current work suggesting that slight increases in protein/gene levels of NRF2 or an increased ratio of NRF2/KEAP1 does not provide sufficient evidence to state that NRF2 activation is responsible for the observed protection[199].

4. Summary and conclusions

As outlined in the previous paragraphs, the murine APAP hepatotoxicity model is clinically relevant but requires an appropriate experimental design to yield results that can be translated to patients. This includes drug treatment after APAP administration and assessment of various mechanistic parameters at multiple time points. Most importantly, the causality of hypothesized mechanisms needs to be directly tested. Adherence to these principles will result in critical mechanistic insight as recently shown with the new antidote 4-methylpyrazole, which protects against APAP hepatotoxicity due to inhibition of CYP2E1[47] and JNK[53]. These findings could then be verified in both human hepatocytes[47] and in human volunteers[52]. The data are the basis for off-label use in overdose patients[200] and the initiation of a phase III clinical trial. If successful, this may be the first new antidote against APAP in 40 years. Importantly, it also is an example how the use of proper experimental design when testing new therapeutic interventions leads to results that can be translated to APAP overdose patients. It is our hope that the discussed pitfalls and recommendations will facilitate the discovery of more clinically relevant interventions.

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Author contributions

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Conflicts of interest

The authors declare no conflicts of interest.

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